Food Chemistry 187 (2015) 120–129

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Development of a quantitative method for the simultaneous analysis of the boar taint compounds androstenone, skatole and indole in porcine serum and plasma by means of ultra-high performance liquid chromatography coupled to high resolution mass spectrometry

J. Wauters [a,*], J. Vanden Bussche [a], K. Verplanken [a], K.M. Bekaert [a], M. Aluwé [b], A. Van den Broeke [b], A. Coussé [c], N. Buys [c], L. Vanhaecke [a,*]

[a] Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium
[b] Institute of Agricultural and Fisheries Research (ILVO), Animal Sciences Unit, Scheldeweg 68, B-9090 Melle, Belgium
[c] KU Leuven University, Science, Engineering and Technology Group, Department of Biosystems (BIOSYST), Division of Gene Technology, Kasteelpark Arenberg 21, B-3001 Heverlee, Belgium

ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form 18 November 2014
Accepted 7 April 2015
Available online 22 April 2015

Keywords:
Pig
Blood
Fat
Off-odour
Orbitrap
LC–MS
Correlation

ABSTRACT

Boar taint is an off-odour occurring while heating meat or fat from boars. A method detecting the three compounds (androstenone, skatole and indole) simultaneously in blood would offer substantial advantages since it would allow monitoring the impact of rearing strategies.

Therefore, a UHPLC-HR-Orbitrap-MS analysis method is optimized and validated for the quantification of these compounds in plasma or serum. Sample pre-treatment involved an extraction with diethylether followed by a centrifugal filtration (30 kDa).

Limits of detection and quantification varied between 0.5 and 1 μg L^{-1} and 2 and 3 μg L^{-1} for the three compounds, respectively. Besides, an excellent repeatability (RSD < 7.6%), within-laboratory reproducibility (RSD < 10.5%), recovery (87–97%) and linearity (R^2 > 0.99) were recorded.

Correlations between serum/plasma and fat levels of the boar taint compounds were positive for skatole (r_{serum} = 0.39 and r_{plasma} = 0.84) and androstenone (r_{serum} = 0.73–0.78 and r_{plasma} = 0.32–0.80).

1. Introduction

The benefits of castration of male piglets have been strongly questioned, not only because of the animals' distress, but also due to substantial economic losses and ecological issues related with raising barrows instead of boars.

Hence, the cessation of castration was recently publically announced and will be obligatory by 2018 (EFSA., 2004). Rearing entire boars has, however, a major disadvantage that is up to now difficult to control. Indeed, boar taint, an off-odour potentially released while heating the meat, is disliked by most consumers who may therefore eventually prefer alternative meat products (Aluwe et al., 2009).

To meet the consumers' expectations, governments, farmers, meat companies and research groups have been and are currently working together to elucidate the physiological mechanisms that are responsible for this taint specific for non-castrated pigs.

A major compound associated with boar taint is the steroidal pig pheromone 5α-androst-16-en-3-one (AEON), which has an unpleasant urine-like and sweaty odour. Upon synthesis in the testis, AEON is released into the bloodstream via the testicular vein and subsequently released in boar saliva or accumulated in adipose tissue due to its lipophilic character (Brooks & Pearson, 1989; Patterson, 1968). Furthermore, predominantly skatole (SK) but also indole (IND), which are both microbial degradation products of tryptophan formed in the intestine by specific bacteria, contribute to boar taint (Claus, Dehnhard, Herzog, Bernalbarragan, & Gimenez, 1993; Deslandes, Gariepy, & Houde, 2001). Their odour perceptions are often described as musty and faecal-like (Claus, Weiler, &

* Corresponding authors. Tel.: +32 9 2647457; fax: +32 9 2647492.
E-mail addresses: Jella.Wauters@ugent.be (J. Wauters), Julie.VandenBussche@ugent.be (J. Vanden Bussche), Kaat.Verplanken@ugent.be (K. Verplanken), Karenbekaert@gmail.com (K.M. Bekaert), Marijke.Aluwe@ilvo.vlaanderen.be (M. Aluwé), Alice.Vandenbroeke@ilvo.vlaanderen.be (A. Van den Broeke), Annelies.Cousse@biw.kuleuven.be (A. Coussé), Nadine.Buys@biw.kuleuven.be (N. Buys), Lynn.Vanhaecke@ugent.be (L. Vanhaecke).

http://dx.doi.org/10.1016/j.foodchem.2015.04.066
0308-8146/

Herzog, 1994). SK and IND are partly absorbed by the intestinal mucosa, distributed via the bloodstream, and finally enriched in adipose tissue and as such also contribute to boar taint (Zamaratskaia & Squires, 2009).

The level of AEON is demonstrated to alter during sexual maturation and is consequently also age and body weight dependent (Zamaratskaia, Babol, Madej, Squires, & Lundström, 2004b; Zamaratskaia & Squires, 2009). These maturation factors are substantially influenced by genetic background. One approach to control boar taint may thus be based on gene identification and manipulation. The SK level, on the other hand, is not only depending on genetic factors, but can also be influenced by diet and environmental factors, which provide another interesting research field possibly allowing to reduce boar taint in a more natural way, e.g. by management measures (Aluwe et al., 2009).

Up to now, the consequences of these interacting factors on the boar taint compounds in fat tissue or blood are studied with a variety or a combination of techniques since only a limited number of authors describe methods for the simultaneous analysis of the indolic compounds and the steroid compound in adipose tissue (Bekaert et al., 2012; Buttinger, Karasek, Verlinde, & Wenzl, 2014; Hansen-Moller, 1994; Rius & Garcia-Regueiro, 2001; Verheyden et al., 2007), and no previous reports on simultaneous measurements of the boar taint compounds in serum or plasma are available. AEON is often determined by ELISA (Claus, Herbert, & Dehnhard, 1997) or gas chromatography coupled to electron-capture detection (De Brabander & Verbeke, 1986), flame ionisation detection (Rius & Garcia-Regueiro, 2001) or mass spectrometry (Berdagué, Viallon, Bonneau, & Ledenmat, 1993), while the indolic compounds skatole and indole are determined by colorimetric methods (Babol, Zamaratskaia, Juneja, & Lundström, 2004) or liquid chromatography coupled to fluorescence detection (Garcia-Regueiro & Rius, 1998). Analysis of the three boar taint compounds simultaneously has been reported using ultra-high performance liquid chromatography coupled to linear trap liquid chromatography or high-resolution mass spectrometry (Bekaert et al., 2012; Buttinger et al., 2014; Verheyden et al., 2007), but also by means of (stable isotope dilution analysis – headspace solid-phase microextraction –) gas chromatography/mass spectrometry (Fischer et al., 2011, 2014; Sørensen & Balling Engelsen, 2014). For liquid matrices such as plasma or serum, which allow generic extraction, liquid chromatography is however the method of choice for separation (Wu, 2001). Besides, the lower concentrations of the boar taint compounds ($\mu g\,L^{-1}$ level) in plasma or serum as compared to adipose tissue ($mg\,kg^{-1}$ level), limit the application of conventional equipment such as single quadrupole mass analysers.

The objective of this study was to develop a quantitative, accurate, robust and fast UHPLC-HRMS-based method that is capable of quantifying the three known boar taint compounds simultaneously in serum or plasma, which would be highly valuable for routine analysis of boar taint at the pre-slaughter stage, e.g. for studies that aim to establish a boar taint decreasing management on the farm. This method was validated according to the guidelines of 2002/657/EC (European Commission Decision, 2002) and ISO 17025 (ISO, 2005).

2. Experimental

2.1. Reagents and chemicals

The reference standards IND (2,3-benzopyrrole), SK (3-methylindole) and AEON (5α-androst-16-ene-3-one) and the internal standards 2-methylindole (2-MID) and androstadienedione (1,4-androstadiene-3,17-dione, ADD) were obtained from Sigma Aldrich (St. Louis, MO, USA). For each compound a stock solution was prepared in methanol at a concentration of $1\,mg\,mL^{-1}$. Working solutions were made for each compound in methanol at a range of 5–100 $ng\,\mu L^{-1}$. Solutions were stored in dark glass bottles at −20 °C.

Reagents were of analytical grade when used for extraction purposes and of MS-grade for UHPLC-MS applications. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Leicestershire, VS), respectively.

Centrifugal filter devices and solid phase extraction (SPE) columns were purchased from Millipore Corporation (Bilerica, US) and Waters Corporation (Milford, US), respectively.

2.2. Samples

2.2.1. Serum samples (study group 1)

The serum samples of entire boars ($n = 32$) were taken from experimental animals at ILVO (Melle, Belgium) after obtaining approval of the ethical committee (EC 2012/180). Samples were acquired by venopunction of the vena jugularis with a Venoject system (Terumo Europe NV, Belgium) coupled to serum collection tubes. Sampling occurred in the morning, one to 13 days before slaughter in a time range of two months (January–February). The boars' ages, all hybrids of Piétrain boars × Rattlerow Seghers sows, varied between 22 and 26 weeks at the time of slaughter. After arrival in the lab (transport on ice), the tubes were centrifuged at 3000*g* during 10 min at 4 °C. The harvested serum was stored at −80 °C until analysis.

In order to obtain reference material, blood of barrows was collected in serum collection tubes during exsanguination at the slaughterhouse and processed as described previously.

2.2.2. Plasma samples (study group 2)

Plasma samples ($n = 79$) were taken from food producing boars of various breeds (Piétrain, Large White, Landrace) which were slaughtered between January 2010 and October 2011 at 18–39 weeks old. Blood was collected into EDTA plasma collection tubes during exsanguination. After arrival in the lab (transport on ice), the tubes were centrifuged at 3000*g* during 10 min at 4 °C. The harvested plasma was stored at −20 °C until analysis.

2.2.3. Paired serum and plasma samples (study group 3)

Serum and plasma samples ($n = 25$) were taken from food producing boars (Landrace; 26–36 weeks old) slaughtered in April 2014. Blood was collected during exsanguination in serum and EDTA plasma collection tubes. Upon arrival at the lab (cooled transport) plasma tubes were centrifuged during 10 min at 1811*g* and 4 °C, while serum tubes were centrifuged at identical speed and temperature but during 30 min. Plasma and serum aliquots were stored at −20 °C until analysis.

2.2.4. Neck fat samples

Back fat samples of the neck region were taken during the slaughter process, packed in plastic bags with (study group 1) or without (study group 2 and 3) vacuum treatment, transported on ice or liquid nitrogen and stored at −80 °C (study group 1 and 3) or −20 °C (study group 2), until analysis. For both study groups, blood samples and fat samples were correctly matched with the help of an additional ear tag attached during exsanguination followed by writing the matching non-erasable pig number on the carcass with a special pencil once the pigs had left the hot baths and scalder prior to evisceration.

2.3. Sample preparation

Aliquots of 1 mL of serum or plasma were transferred into 15 mL tubes for analysis. Each sample was spiked with 20 μL of internal standard mixture (2-MID and ADD at 2.5 ng μL^{-1}). Next, 2 mL of diethylether was added and the sample was rigorously vortexed for 1 min and centrifuged at 13,500*g* for 5 min at 4 °C to obtain phase separation. Subsequently, the extract was dried under a gentle stream of nitrogen at 30 °C, redissolved in 500 μL of methanol and rigorously vortexed. Samples were further purified by filtration over a Microcon 30 kDa centrifugal filter device (Amicon® Ultra-0,5, 30 kDa) by centrifugation at 13,500*g* for 10 min at 4 °C, and 75 μL of this extract was diluted with 75 μL of 0.05% formic acid in water and passed to the HPLC inserts for analysis.

2.4. Instrumentation

The UHPLC system consisted of a Thermo Fisher (San José, CA, USA) Accela UHPLC pumping system coupled to an Accela Autosampler and Degasser.

Chromatographic separation was achieved using reversed phase chromatography with gradient elution. Separation of the compounds was carried out on a Hypersil Gold column (1.9 μm, 50 mm × 2.1 mm ID) (Thermo Fisher Scientific). The mobile phase consisted of a mixture of methanol and 0.05% formic acid, pumped at a flow rate of 0.3 mL min^{-1}. Optimized separation of the compounds was obtained using a linear gradient (Supplementary Table 1) (Bekaert et al., 2012).

Mass spectrometric analysis was carried out using an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) fitted with an atmospheric-pressure chemical ionisation source (APCI) operated in the positive ion mode. The optimal ionisation source working parameters can be consulted in Bekaert et al. (2012) (Supplementary Table 2). A scan range of *m*/*z* 100–500 was chosen and the resolution was set at 50,000 full width half maximum (FWHM) at 2 Hz (2 scans per second). The automatic gain control (AGC) target was set at high dynamic range (3×10^6) and the maximum injection time was 500 ms.

Initial instrument calibration was done by infusing calibration mixtures for the positive and negative ion modes (Thermo Fisher Scientific). The positive calibration mixture included caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark® 1621, while the negative calibration solution comprised of sodium dodecyl sulphate, sodium taurocholate and Ultramark® 1621. These compounds were dissolved in a mixture of acetonitrile, water and methanol, and both mixtures were infused using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific). The option of "all-ion fragmentation" using the High Energy Collision Dissociation (HCD) cell was turned off. The fore vacuum, high vacuum and ultra high vacuum were maintained at approximately 2 mbar, from 10^{-5} to 3×10^{-5} and below 8×10^{-10} mbar, respectively.

Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific).

2.5. Quality assurance

Prior to the sample analysis, a standard mixture (2 ng on column) of the target compounds was injected to evaluate the operational conditions of the chromatographic devices. To every sample, a mixture of internal standards (2-MID and ADD) was added at a concentration of 2.5 ng μL^{-1}, prior to extraction.

Analytes were identified based on their retention time relative to that of the internal standard and their accurate mass (Table 1). For quantification purposes, ten-point-based matrix-matched calibration curves were prepared by spiking serum or plasma samples with a standard mixture of indole, skatole and androstenone obtaining ten concentrations (0, 0.5, 2.5, 5, 12.5, 25, 50, 100, 150 and 200 μg L^{-1}). The internal standards 2-MID and ADD were added at a concentration of 2.5 μg μL^{-1}.

Table 1
Compound specific MS parameters for the different boar taint compounds (IS = internal standard, RT = retention time).

Analyte	Ion mode	IS	RT (min)	Accurate mass (*m*/*z*)	Mean mass error (ppm)	C13 ratio (%)	Ratio error (%)
IND	+	2-MID	1.32	118.06513	−1.03	8.65	5.90
SK	+	2-MID	2.35	132.08078	−1.09	9.73	3.99
AEON	+	ADD	5.35	273.22129	−2.69	20.55	8.50

2.6. Limits of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on the outcome of 7-point calibration curves in matrix in a range of 0–50 μg L^{-1}.The LOD and LOQ were calculated as the concentrations which generate chromatographic peaks with a signal-to-noise ratio of 3:1 and 10:1, respectively, according to ISO 17025 (ISO, 2005).

2.7. Accuracy and precision

As no certified reference material was available, the recovery was assessed by spiking serum of barrows, containing low background concentrations of the analytes of interest. Serum samples were spiked at a concentration of 5, 10 and 20 μg L^{-1} for all compounds in six replicates.

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Both validation parameters were evaluated by calculating the relative standard deviations (RSD, %). For evaluating the repeatability, three series of six replicates of three samples, at a concentration of respectively 5, 10 and 20 μg L^{-1}, were analysed. These three series of analyses were carried out on different occasions by the same operator under repeatable conditions. A similar set-up (4 series of six replicates at the three mentioned concentrations) was elaborated to evaluate the within-laboratory reproducibility. However, the series' replicates were now executed by different lab technicians on another day, whereby environmental conditions consequently altered.

2.8. Correlation between fat and serum analysis

Parallel analyses of plasma ($n = 79 + 25$) or serum ($n = 32 + 25$) samples and adipose tissue samples ($n = 79 + 32 + 25$) obtained from boars were performed to evaluate the correlation between the concentrations of the boar taint compounds in the two matrices. Adipose tissue analysis was performed according to Bekaert et al. (2012). In short, liquid fat (microwave) was mixed with methanol before heath- and subsequent freeze-treatment. Following centrifugation, the extract was prepared for solid phase extraction (SPE) resulting in a final extract which was diluted with 0.05% formic acid before injection onto the U-HPLC system. Additionally, paired serum and plasma concentrations ($n = 25$) of the boar taint compounds were correlated.

SPSS version 22.0 was used for statistical analysis, including the assessment of a linear relationship between serum/plasma and fat

boar taint compound concentrations. Significant correlations ($p < 0.05$) were expressed using Pearson's correlation coefficient (r). The descriptive statistics (mean with standard deviation, median and range) of the boar taint compound concentrations in plasma or serum and fat concentrations of the animals included in the three studied populations are also reported.

3. Results and discussion

3.1. Development of sample pre-treatment

As previously documented, information on extraction and clean-up (solvents, SPE columns, ...) of boar taint compounds was intensively gathered at the lab during the optimization of the boar taint compound detection in adipose tissue (Bekaert et al., 2012). Accordingly, relevant information was extrapolated to serum pre-treatment and further optimization was restricted to minor changes in the clean-up and concentration step.

In more detail, three clean up procedures, following liquid liquid extraction (LLE) with diethylether, were compared for serum by evaluating the outcome of three (for each procedure) ten-point-based matrix-matched calibration curves. The first two conditions required an evaporation step after LLE, followed by reconstitution in methanol, whereafter either microcon filtration (Amicon® Ultra-0,5, 30 kDa) or solid phase extraction (SPE) (Oasis HLB, 3 ml, 60 mg) was performed. The evaporation step was omitted in a third condition with LLE instantly followed by SPE. Based on the linearity of the calibration curves, the inclusion of an evaporation step revealed to be mandatory and both SPE and ultrafiltration scored equally well ($R^2 \geqslant 0.99$). However, the RSD below 11% for ultrafiltration compared to 35% for SPE encouraged the use of the ultrafiltration clean up procedure in future analysis.

3.2. UHPLC and MS parameters

The analytical method was adopted from Bekaert et al. (2012) who developed the procedure for the simultaneous quantification of three boar taint compounds in fat tissue. A quick overview of the most relevant U-HPLC and MS parameters can be consulted in Tables 1 and 2.

3.3. Method validation

The Commission Decision 2002/657/EC (European Commission, 2002) and ISO17025 (ISO, 2005) were used as guidelines for validation of the developed detection method for IND, SK and AEON.

Levels of these boar taint compounds in plasma or serum ($\mu g\,L^{-1}$) are much lower compared the levels in fat ($mg\,kg^{-1}$). Minimum and maximum plasma and serum levels for entire male pigs between 1–240, 0.55–75 and 1–15 $\mu g\,L^{-1}$ for AEON, SK and IND, respectively, have been reported (Chen, Zamaratskaia, Andersson, & Lundström, 2007; Tuomola, Harpio, Knuuttila, Mikola, & Lovgren, 1997; Tuomola, Harpio, Wirta, & Lovgren, 2002; Tuomola, Vahva, & Kallio, 1996; Zamaratskaia, Babol, Andersson, & Lundström, 2004a).

Appropriate internal standards were thus selected, capable of anticipating fluctuations in signal intensity upon extraction of boar taint compounds from plasma or serum samples. Verheyden et al. (2007) used 2-methylindole (2-MID) as internal standard and this compound was found satisfactory in this study for the indolic compounds as well. Androstadienedione (ADD) was added as an additional internal standard for the steroid compound AEON.

Validation was performed on serum and additional testing was performed on plasma, including evaluation of linearity, LOD and LOQ.

3.3.1. Specificity

The specificity of the method could be demonstrated by analysis of blank serum samples and samples fortified with each analyte separately at 20 $\mu g\,L^{-1}$. Since true blank serum samples are not available (Hansen-Moller, 1994), serum from barrows, which contains very low background concentrations of the analytes of interest, was selected to validate the method. For each analyte spiked, the chromatograms showed a significant increase in peak area intensity at the specific retention time of the compounds, taking a signal-to-noise ratio of at least 3 into account. No other matrix substances interfered at this exact retention time although non-interfering peaks were noticed at different retention times for AEON in the blank chromatogram (Fig. 1). As a result, the developed method was found to be specific for IND, SK and AEON in the presence of matrix compounds.

3.3.2. Selectivity

Analytes were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the internal standard. In addition, the accurate mass of the ions was taken into account when the chromatographic peak of interest had a signal-to-noise ratio of at least 3. A maximum mass deviation of 3 ppm was allowed within this study (Table 1).

3.3.3. Linearity

The linearity of the developed method was evaluated by preparing calibration curves in matrix for the different compounds. The blank samples were fortified with concentrations ranging from 0.5 to 200 $\mu g\,L^{-1}$. For both plasma and serum, linearity and lack-of-fit were assessed by building an univariate linear regression model (SPSS version 22.0) with the (3 times replicated) calibration

Table 2
Method recovery and precision of the developed method for the three boar taint compounds, in serum.

Analyte	Nominal concentration ($\mu g\,L^{-1}$)	Mean recovery Mean ± SD (n = 18)	Precision		
			Repeatability		Within-laboratory reproducibility
			Mean ± SD (n = 18)	RSD (%) (n = 18)	RSD (%) (n = 24)
IND	5	90 ± 7	4.5 ± 0.3	7.5	8.7
	10	93 ± 7	9.3 ± 0.7	7.1	7.6
	20	89 ± 4	17.8 ± 0.7	4.0	10.4
SK	5	90 ± 6	4.5 ± 0.3	6.6	6.1
	10	93 ± 5	9.3 ± 0.5	5.6	5.8
	20	90 ± 2	18.1 ± 0.3	1.8	1.8
AEON	5	97 ± 4	4.9 ± 0.2	4.2	6.4
	10	92 ± 4	9.2 ± 0.4	4.7	4.8
	20	87 ± 2	17.3 ± 0.4	2.5	3.1

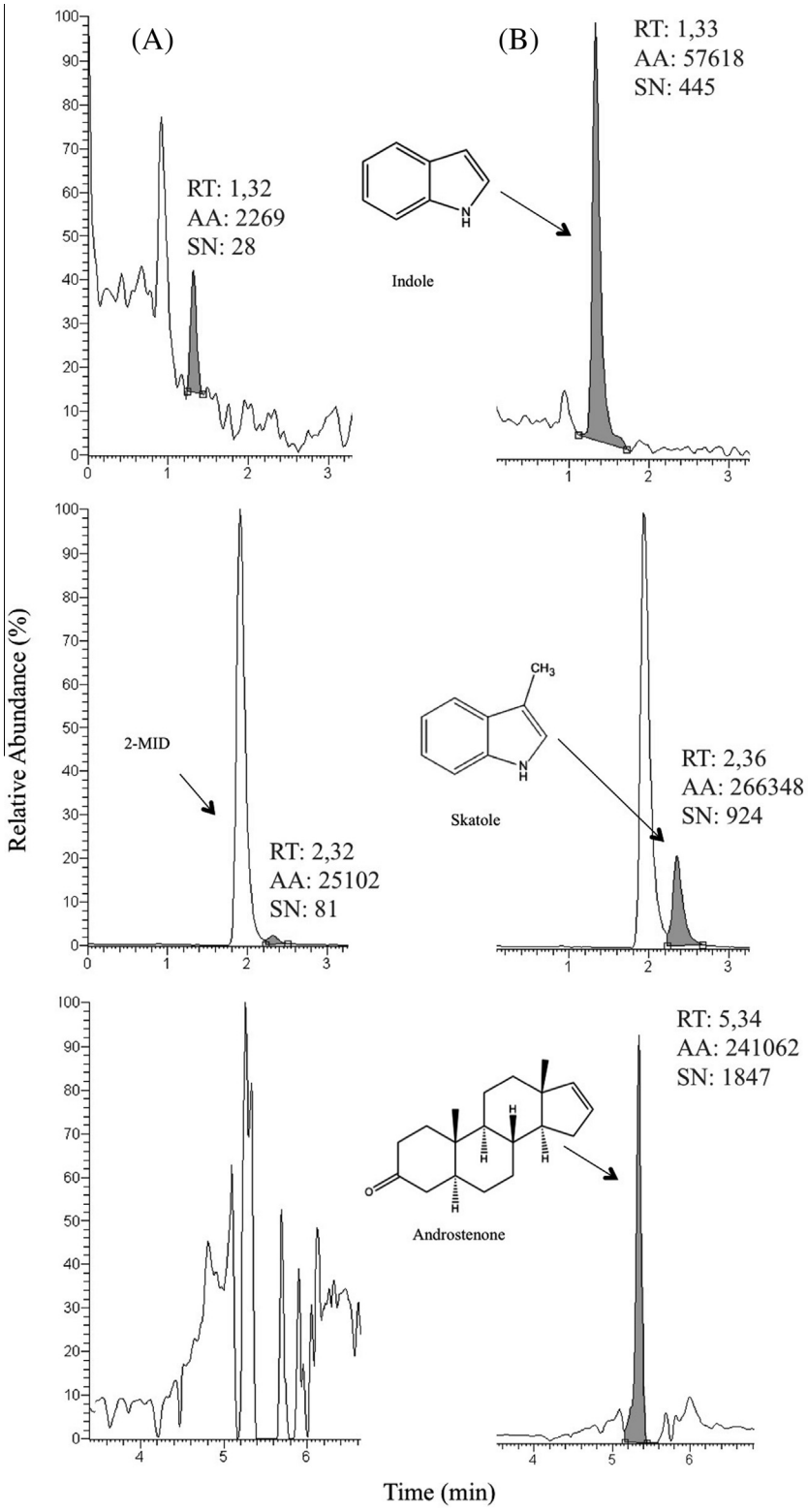

Fig. 1. Chromatogram of a blank serum sample (A) and a sample fortified with 20 μg L^{-1} of IND, SK and AEON (B) analysed on the U-HPLC-Exactive™ (RT: retention time; MA: area; SN: signal-to-noise ratio).

concentrations as independent variable and the respective area ratios (e.g. area compound/area respective internal standard) as dependent variable. The resulting regression model equations were reported to be all linear (*F*-test; $p < 0.05$; $R^2 \geqslant 0.99$). Model validity was additionally confirmed by absence of any lack-of-fit (95% confidence interval).

3.3.4. Accuracy and precision

The recoveries obtained with this analytical method were satisfactory (Table 2). As presented in Table 2, the calculated RSD values (RSD < 7.6%) were below 15%, indicating a good repeatability according to European Criteria 2002/657/EC (European Commission, 2002). For the within-laboratory reproducibility, the

calculated RSD values (RSD < 10.5%) were below 20%, indicating good precision of the method (European Commission, 2002). Moreover, these validation parameters proved to perform equally well compared to other detection methods in serum or plasma such as HPLC-fluorescence detection (RSD ⩽ 12.0%) (Brunius & Zamaratskaia, 2012; Tuomola et al., 1996), LC-MS/MS (RSD ⩽ 11.0%) (Chen et al., 2010) and a time-resolved fluoroimmunoassay (RSD ⩽ 8.1%) (Tuomola et al., 1997).

3.3.5. Limit of detection and limit of quantification

Limits of detection (LOD) and quantification (LOQ) were determined based on the outcome of ten-point calibration curves in matrix in a range of 0.5–200 μg L^{-1}. The LOD and LOQ was respectively 0.5 and 2.0 μg L^{-1} for IND and SK and 1.0 and 3.0 μg L^{-1} for AEON (Fig. 2). The respective levels did not differ between serum or plasma. These LOD and LOQ are noticeably lower compared to fat if a similar detection technique is considered (Bekaert et al., 2012), while for SK and IND, but not for AEON, slightly lower limits are reported by Fischer et al. (2011) applying a stable isotope dilution analysis – headspace solid phase microextraction – gas chromatography/mass spectrometry. However, considering only serum or plasma specific detection techniques, the limits of this study are in accordance, although comparison is not straightforward due to aberrant criteria of determination of these limits (Brunius & Zamaratskaia, 2012; Chen et al., 2010; Haugen, Brunius, & Zamaratskaia, 2012; Tuomola et al., 1997).

3.4. Correlation with fat analysis

To evaluate the presence or absence of a positive or negative linear relationship between IND, SK and AEON concentrations in serum or plasma and fat, the Pearson's correlation coefficient was used.

For all statistical analyses (blood and fat), values below the LOD were replaced by an arbitrary value equal to LOD/$\sqrt{2}$ (Croghan & Egeghy, 2003). The descriptive statistics of this study can be consulted as Supplementary information in Supplementary Table 3. To facilitate literature review, both mean (standard deviation) and median (range) concentrations are reported. Similar mean and median concentrations were previously described for serum, plasma and fat (Chen et al., 2007; Tuomola et al., 1996, 1997; Zamaratskaia, Babol, Madej, Squires, & Lundström, 2004b).

In this study, serum and/or plasma and their paired neck fat boar taint levels were studied by the analysis of three different study groups. In none of the study groups, a significant fat-blood correlation could be demonstrated for IND (Fig. 3 and Supplementary Fig. 1). Nevertheless, serum and plasma IND levels were obviously correlated despite recording only 5 sample concentrations above the LOD (r_{IND} = 0.72; p < 0.001). In study group 1, the serum levels for SK and particularly for AEON were clearly positively correlated with the corresponding fat levels, resulting in correlation coefficients of r_{serum} = 0.39 (p < 0.05) and r_{serum} = 0.73 (p < 0.001), respectively. These correlation coefficients are in accordance with those obtained for study group 3 in which serum, plasma and fat were sampled from the same pigs. A strong correlation was observed between serum and fat and plasma and fat for AEON with r_{serum} = 0.78 (p < 0.001) and r_{plasma} = 0.80 (p < 0.001) (Supplementary Fig. 3). For SK, the Pearson's correlation coefficient lacked significance with r_{serum} = 0.31 (p = 0.13) and r_{plasma} = 0.22 (p = 0.28) (Supplementary Fig. 2). SK and AEON concentrations matched significantly between serum and plasma as illustrated by correlations coefficients of r_{SK} = 0.97 (p < 0.001) and r_{AEON} = 0.95 (p < 0.001) (Supplementary Figs. 2 and 3). The excellent correlation between serum and plasma concentrations, in particular for SK and AEON, but also for IND, does not only imply an interesting scientific insight in blood-derived boar taint levels, but also confirms the applicability of this analytical method for both matrices. In this context, the plasma-fat correlation coefficients derived from study group 2 were surprisingly aberrant, as the strongest relationship was not observed for AEON, but for SK, with a correlation coefficient of 0.84 (p < 0.001) for SK and a rather poor result for AEON (r_{plasma} = 0.32; p < 0.05) (Fig. 3). The observed boar taint compound ranges were generally substantially wider in this study group, particularly for fat (higher maximum limits for all compounds, lower minimum limits for AEON), but for plasma as well if compared to both other study groups. Additionally, serum level ranges of IND and SK and fat level ranges of SK (upper limit) and AEON (lower limit) did not match between study group 1 and 3 with the narrowest ranges for the latter. Due to the discrepancies between the three study groups, an additional correlation study was performed with exclusion of data points below or above the minimum respectively maximum values recorded for study group 3 (Supplementary Fig. 4 and Supplementary Table 4). This resulted in significant and comparable correlation coefficients for IND between plasma and fat (Study group 2 (n = 66), r_{plasma} = 0.36, p < 0.05) and serum and fat (study group 1 (n = 31), r_{serum} = 0.43, p < 0.05). Lower non-significant correlation coefficients were obtained in study group 3 (n = 25; respectively, r_{plasma} = 0.26, p = 0.21; r_{serum} = 0.27, p = 0.19).

With a reduction of 8 plasma-fat concentration pairs (n = 71), the aberrant correlation coefficient of r_{plasma} of 0.84 for SK dramatically decreased to r_{plasma} = 0.29 (p < 0.05), a value which is more aligned with the overall results of study group 1 (n = 32, r_{serum} = 0.39, p < 0.05; n = 22, r_{serum} = 0.26, p = 0.25) and study group 3 (n = 25; r_{serum} = 0.31, p = 0.13 and r_{plasma} = 0.22, p = 0.28). Correlations for AEON remained aberrant and were only slightly influenced with a lacking statistical significance in study group 2 (n = 40; r_{plasma} = 0.20, p = 0.22), whereas the correlation coefficient slightly increased between serum and fat samples (study group 1, n = 14, r_{serum} = 0.85, p < 0.001).

Reduction of the dataset of study group 1 and 2 towards boar taint compound levels fitting within the ranges achieved for study group 3, resulted in comparable results for IND and SK in the three study groups. However, for AEON, which is also characterised by a broader and more equally-spread concentration range compared to both other compounds, the correlation coefficient of group 2 remained unexpectedly low (n = 40; r_{plasma} = 0.20, p = 0.22). To this study group, however, substantial variations in seasonal sampling (winter to autumn), included breeds (Piétrain, Large White, Landrace), origin (several breeding/fattening farms), slaughtering conditions (slaughterhouses) and age (18–39 weeks) can be attributed, which is not or hardly the case in both other study groups (short one-seasonal sampling-timeframe, one breed, limited age-range). Consequently, the non-uniformity of the study-design related factors in study group 2 might have confounded the outcomes of correlations, as it is very likely that different coefficients of correlation are valid depending on the subpopulation. Factors such as seasonal aspects, carcass characteristics, genetic background, age (in relation to puberty or weight), transport towards the slaughterhouse and environmental conditions were indeed previously highlighted to influence both the indolic boar taint compounds and AEON in both blood (plasma/serum) and fat (Aluwe et al., 2011, 2009; Andresen, 1976; Bonneau, 1987; Knorr, Wahl, Sharifi, & Mörlein, 2013; Rasmussen & Zamaratskaia, 2014; Rowe et al., 2014; Wesoly, Jungbluth, Stefanski, & Weiler, 2015; Zamaratskaia et al., 2004a,b).

The season and the genetic profile (breed) are thus two probable factors responsible for a major discrepancy between the second study group and both others. Although statistical evaluation of any interactions between these and other factors is out of the scope of this study, Supplementary Fig. 5 reveals limited additional information on study group 2 after clustering the AEON data in plasma

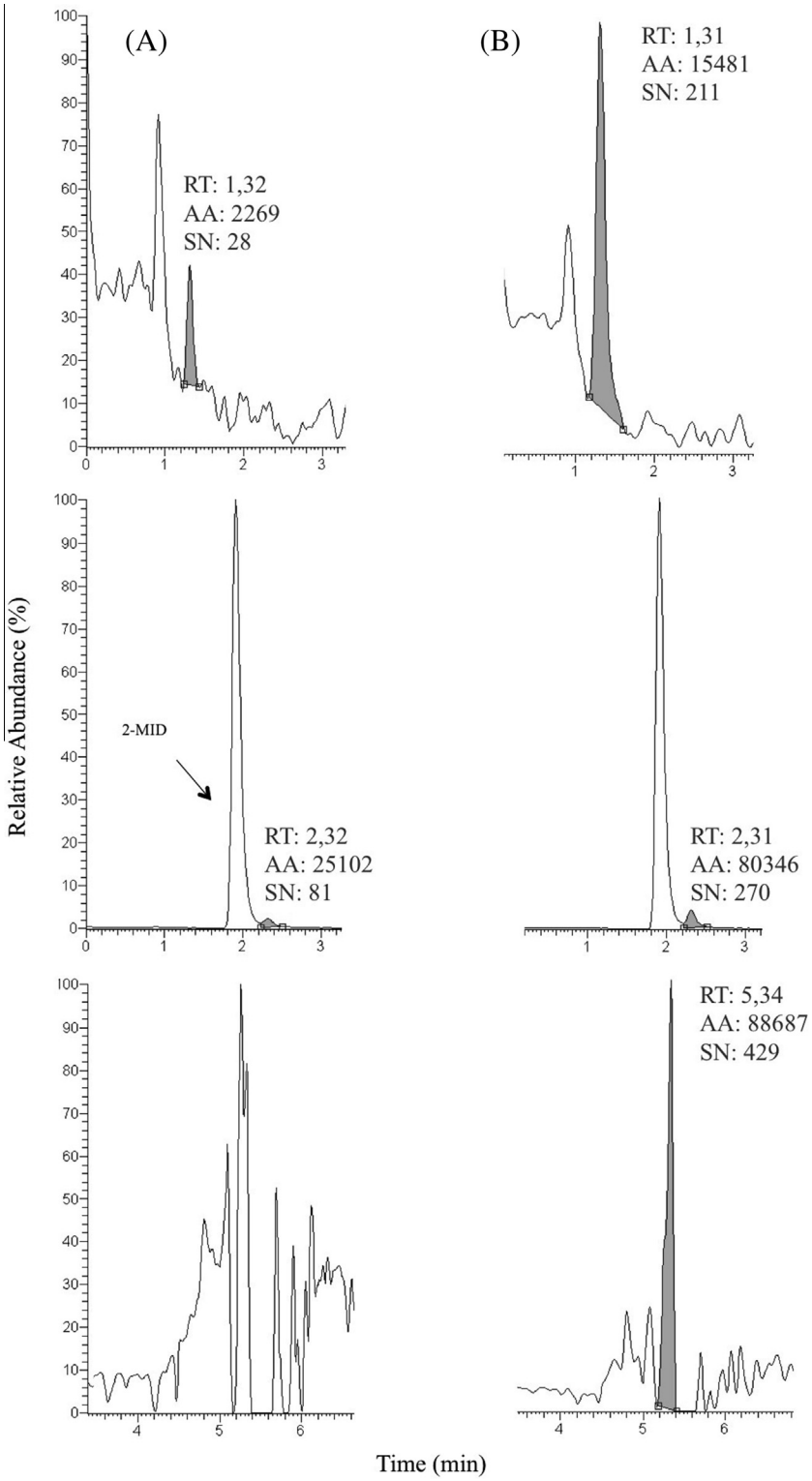

Fig. 2. Chromatogram of a blank serum sample (A) and a sample spiked at the limit of quantification (IND and SK: 2 μg L^{-1}; AEON: 3 μg L^{-1} (B)) analysed on the U-HPLC-Exactive™ (RT: retention time; AA/MA: area; SN: signal-to-noise ratio).

and fat based on respectively season or breed. The correlation between plasma and fat concentrations of AEON seems indeed to be prone to variations in the latter factors as varying (significant and non-significant) correlation coefficients were achieved for samples taken in different seasons and from different breeds. An overview of the most relevant descriptives is added in Supplementary Table 5.

Finally, conclusions on serum/plasma-fat correlations have never been straightforward as some authors claimed a complete absence of correlations (Bonneau, Meusydessolle, Leglise, & Claus, 1982; Lundström, Malmfors, Hansson, Edqvist, & Gahne, 1978; Malmfors, Lundström, & Hansson, 1978) while others illustrated remarkably high (up to $r = 0.91$) correlations (Andresen, 1976; Babol, Squires, & Lundström, 1999; Booth, Williamson, &

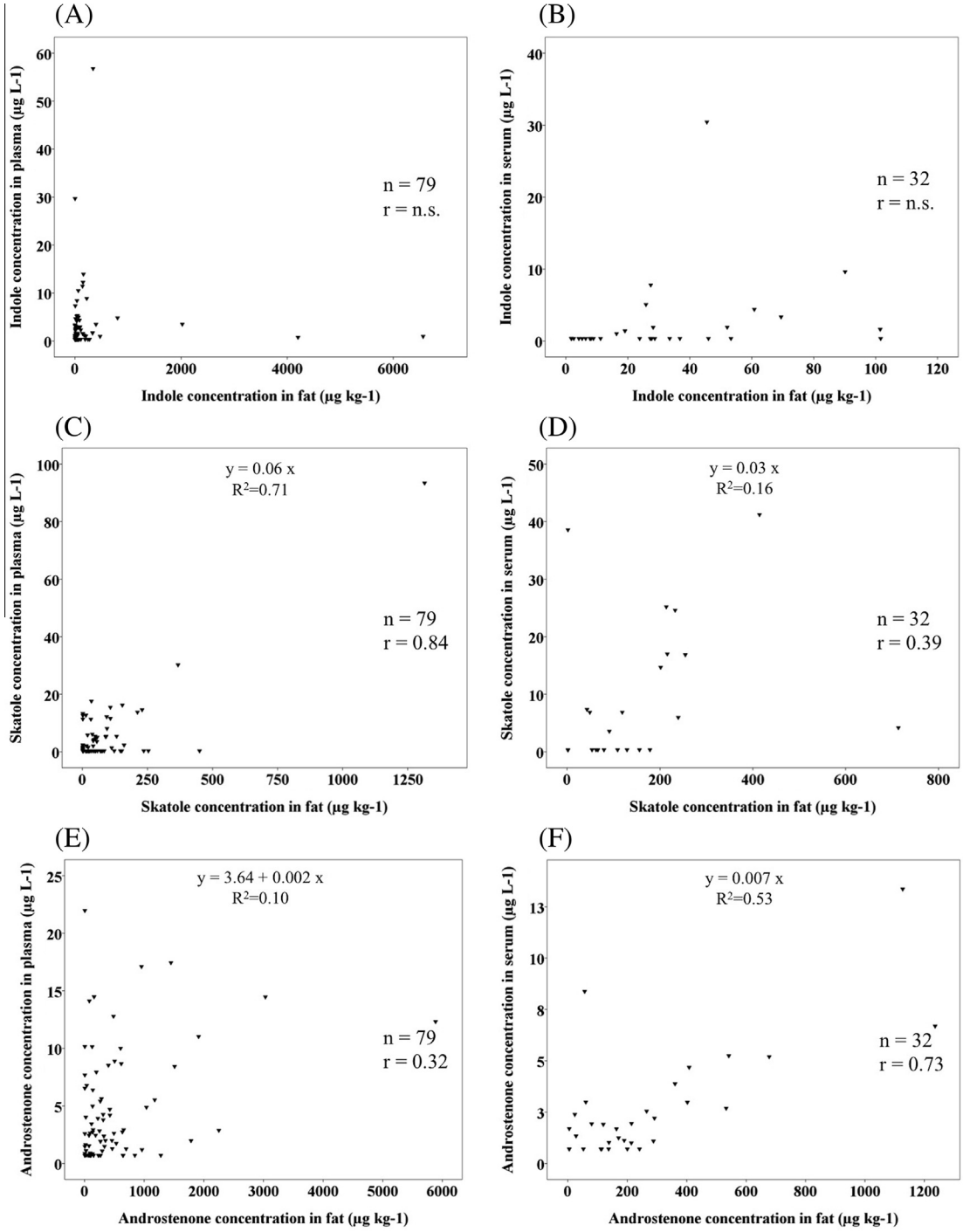

Fig. 3. Scatterplots for the three boar taint compounds in plasma and fat (IND (A), SK (C) and AEON (E)) (study group 2) and serum and fat (IND (B), SK (D), AEON (F)) (study group 1), with significant regression equations (n = number of animals included, r = Pearson's correlation coefficient, R^2 = determination coefficient; n.s. = not significant).

Patterson, 1986; Tuomola et al., 1996, 2002). In these studies, different outcomes may have several scientific explanations. Besides the previously discussed influence of study-design related factors, different mechanism of detection, between studies but more importantly between fat and blood analyses within one study, may have another important impact.

One of the great advantages of this study, results from the fact that both fat and serum/plasma levels are gathered by applying similar techniques. Besides a matrix-adapted extraction and clean-up of the samples, any other analytical characteristics were kept identical (standard addition, instrumental parameters, ...) (Bekaert et al., 2012). Logically, we can assume that fat and blood levels acquired with these complementary techniques will result in correlation coefficients that fit closer compared with earlier reported values commonly based on combinations of HPLC and colorimetric techniques. Recently, some research groups suggested the use of boar taint blood levels as predictive values for boar taint in meat, based on the strong serum/plasma-fat correlation reported in their study (Chen et al., 2010; Zamaratskaia et al., 2004a). However, as the discrepancy between several studies currently only leads to inconclusiveness, more research is warranted to properly investigate these correlations by means of comparable

analytical techniques for both blood and fat matrices. Moreover, it should be highlighted that such studies should not only be based on analytical boar taint outcomes. Indeed, the 'analytical' presence of boar taint in the blood should not only be correlated to corresponding fat levels, but more importantly, it should be sensory confirmed in the meat (e.g. heated neck fat assessed by trained expert panel) in order to validate the possible predictive value of boar taint blood levels.

If future research would subsequently reveal a close relationship between the levels of boar taint in sera and the intensity of off-odour in meat for consumption, serologic determination of boar taint compounds may provide a new approach in the prevention of economic meat losses that occur between farm and fork. Meanwhile this technique is the first in his kind to allow simultaneous analysis of the three boar taint compounds in sera and offers therefore a new valuable tool for any study designed to elucidate or control the release and accumulation of boar taint during pig rearing.

4. Conclusions

For the first time, a robust U-HPLC-HR-MS method was successfully validated for the simultaneous quantification of AEON, IND, SK in pig plasma or serum. This method combines a generic pretreatment of the samples with a sensitive and specific detection with excellent performance allowing the detection of the three boar taint compounds in both plasma and serum, within the concentration range of interest. Positive linear correlations could be observed between SK and AEON levels in serum or plasma and fat.

Acknowledgements

The authors would like to thank M. Naessens, D. Stockx, I. Opsteyn, J. Goedgebuer, G. Vanantwerpen, L. De Wilde and V. Vercruysse for their technical assistance and N. Zwaenepoel (slaughterhouse Goossens, Waregem) for the opportunity to sample boars.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.04.066.

References

Aluwe, M., Bekaert, K. M., Tuyttens, F. A. M., Vanhaecke, L., De Smet, S., De Brabander, H. F., et al. (2011). Influence of soiling on boar taint in boars. *Meat Science, 87*(3), 175–179.

Aluwe, M., Millet, S., Nijs, G., Tuyttens, F. A., Verheyden, K., De Brabander, H. F., et al. (2009). Absence of an effect of dietary fibre or clinoptilolite on boar taint in entire male pigs fed practical diets. *Meat Science, 82*(3), 346–352.

Andresen, O. (1976). Concentrations of fat and plasma 5-alpha-androstenone and plasma testosterone in boars selected for rate of body-weight gain and thickness of back fat during growth, sexual-maturation and after mating. *Journal of Reproduction and Fertility, 48*(1), 51–59.

Babol, J., Squires, E. J., & Lundström, K. (1999). Relationship between metabolism of androstenone and skatole in intact male pigs. *Journal of Animal Science, 77*(1), 84–92.

Babol, J., Zamaratskaia, G., Juneja, R. K., & Lundström, K. (2004). The effect of age on distribution of skatole and indole levels in entire male pigs in four breeds: Yorkshire, Landrace, Hampshire and Duroc. *Meat Science, 67*(2), 351–358.

Bekaert, K. M., Vanden Bussche, J., Francois, S., Tuyttens, F. A. M., De Brabander, H. F., Vandendriessche, F., et al. (2012). A validated ultra-high performance liquid chromatography coupled to high resolution mass spectrometry analysis for the simultaneous quantification of the three known boar taint compounds. *Journal of Chromatography A, 1239*, 49–55.

Berdagué, J. L., Viallon, C., Bonneau, M., & Ledenmat, M. (1993). *Measurement and prevention of boar taint in entire male pigs*. Paris.

Bonneau, M. (1987). Effects of age and live weight on fat 5α-androstenone levels in young boars fed two planes of nutrition. *Reproduction Nutrition Development, 27*(2A), 413–422.

Bonneau, M., Meusydessolle, N., Leglise, P. C., & Claus, R. (1982). Relationships between fat and plasma androstenone and plasma testosterone in fatty and lean young boars during growth and after Hcg stimulation. *Acta Endocrinologica, 101*(1), 119–128.

Booth, W. D., Williamson, E. D., & Patterson, R. L. S. (1986). 16-Androstene steroids in the submaxillary salivary-gland of the boar in relation to measures of boar taint in carcasses. *Animal Production, 42*, 145–152.

Brooks, R. I., & Pearson, A. M. (1989). Odor thresholds of the C19-delta-16-steroids responsible for boar odor in pork. *Meat Science, 25*(1), 11–19.

Brunius, C., & Zamaratskaia, G. (2012). A modified high performance liquid chromatographic method for simultaneous quantification of skatole and indole in porcine plasma. *Acta Veterinaria Brno, 81*(2), 153–158.

Buttinger, G., Karasek, L., Verlinde, P., & Wenzl, T. (2014). In house validation of a reference method for the determination of boar taint compounds by LC-MSMS. *JRC Validated Methods, Reference Methods and Measurements*. http://dx.doi.org/10.2787/88600.

Chen, G., Ren, L., Yang, S. M., Qiu, J., Kamal-Eldin, A., & Lundström, K. (2010). Determination of androstenone levels in porcine plasma by LC–MS/MS. *Food Chemistry, 122*(4), 1278–1282.

Chen, G., Zamaratskaia, G., Andersson, H. K., & Lundström, K. (2007). Effects of raw potato starch and live weight on fat and plasma skatole, indole and androstenone levels measured by different methods in entire male pigs. *Food Chemistry, 101*(2), 439–448.

Claus, R., Dehnhard, M., Herzog, A., Bernalbarragan, H., & Gimenez, T. (1993). Parallel measurements of indole and skatole (3-methylindole) in feces and blood-plasma of pigs by Hplc. *Livestock Production Science, 34*(1–2), 115–126.

Claus, R., Herbert, E., & Dehnhard, M. (1997). Comparative determination of the boar taint steroid androstenone in pig adipose tissue by a rapid enzyme immunoassay and an HPLC-method. *Archiv Fur Lebensmittelhygiene, 48*(2), 27–30.

Claus, R., Weiler, U., & Herzog, A. (1994). Physiological-aspects of androstenone and skatole formation in the boar – A review with experimental-data. *Meat Science, 38*(2), 289–305.

Croghan, C. W., & Egeghy, P. P. (2003). *Methods of dealing with values below the limit of detection using SAS*. Sint Petersburg: In Southern SAS User Group.

De Brabander, H. F., & Verbeke, R. (1986). Quantitative determination of androstenone in pig adipose tissue. *Journal of Chromatography, 363*(2), 293–302.

Deslandes, B., Gariepy, C., & Houde, A. (2001). Review of microbiological and biochemical effects of skatole on animal production. *Livestock Production Science, 71*(2–3), 193–200.

EFSA (2004). *The EFSA Journal, 91*, 1–99.

European Commission Decision 2002/657/EC (2002), 8.

Fischer, J., Elsinghorst, P. W., Bucking, M., Tholen, E., Petersen, B., & Wust, M. (2011). Development of a candidate reference method for the simultaneous quantitation of the boar taint compounds androstenone, 3 alpha-androstenol, 3 beta-androstenol, skatole, and indole in pig fat by means of stable isotope dilution analysis-headspace solid-phase microextraction-gas chromatography/mass spectrometry. *Analytical Chemistry, 83*(17), 6785–6791.

Fischer, J., Haas, T., Leppert, J., Schulze Lammers, P., Horner, G., Wüst, M., et al. (2014). Fast and solvent-free quantitation of boar taint odorants in pig fat by stable isotope dilution analysis-dynamic headspace-thermal desorption-gas chromatography/time-of-flight mass spectrometry. *Food Chemistry, 158*, 345–350.

Garcia-Regueiro, J. A., & Rius, M. A. (1998). Rapid determination of skatole and indole in pig back fat by normal-phase liquid chromatography. *Journal of Chromatography A, 809*(1–2), 246–251.

Hansen-Moller, J. (1994). Rapid high-performance liquid chromatographic method for simultaneous determination of androstenone, skatole and indole in back fat from pigs. *Journal of Chromatography B: Biomedical Sciences and Applications, 661*(2), 219–230.

Haugen, J. E., Brunius, C., & Zamaratskaia, G. (2012). Review of analytical methods to measure boar taint compounds in porcine adipose tissue: The need for harmonised methods. *Meat Science, 90*(1), 9–19.

ISO17025. (2005).

Knorr, C., Wahl, H., Sharifi, A. R., & Mörlein, D. (2013). *Different preslaughter fasting periods and their effects on boar taint compounds in finishing pigs*. Nantes, France: In Annual Meeting of the European Association for Animal Production.

Lundström, K., Malmfors, B., Hansson, I., Edqvist, L. E., & Gahne, B. (1978). 5-alpha-androsterone and testosterone in boars – Early testing with Hcg, sexual stimulation and diurnal-variation. *Swedish Journal of Agricultural Research, 8*(3), 171–180.

Malmfors, B., Lundström, K., & Hansson, I. (1978). Interrelations between boar taint, 5-alpha-androsterone and fatty-acid composition in pigs. *Swedish Journal of Agricultural Research, 8*(3), 161–169.

Patterson, R. L. S. (1968). 5alpha-androst-16-ene-3-1 – Compound responsible for taint in boar fat. *Journal of the Science of Food and Agriculture, 19*(1), 31–38.

Rasmussen, M. K., & Zamaratskaia, G. (2014). Regulation of porcine hepatic cytochrome P450 – Implication for boar taint. *Computational and Structural Biotechnology Journal, 11*, 106–112.

Rius, M. A., & Garcia-Regueiro, J. A. (2001). Skatole and indole concentrations in Longissimus dorsi and fat samples of pigs. *Meat Science, 59*(3), 285–291.

Rowe, S. J., Karacaören, B., de Koning, D.-J., Lukic, B., Hastings-Clark, N., Velander, I., et al. (2014). Analysis of the genetics of boar taint reveals both single SNPs and regional effects. *BioMed Central Genomics, 15*, 424.

Sørensen, K. M., & Balling Engelsen, S. (2014). Measurement of boar taint in porcine fat using a high-throughput gas chromatography – Mass spectrometry protocol. *Journal of Agricultural and Food Chemistry, 62*, 9420–9427.

Tuomola, M., Harpio, R., Knuuttila, P., Mikola, H., & Lovgren, T. (1997). Time-resolved fluoroimmunoassay for the measurement of androstenone in porcine serum and fat samples. *Journal of Agricultural and Food Chemistry, 45*(9), 3529–3534.

Tuomola, M., Harpio, R., Wirta, E. R., & Lovgren, T. (2002). Monitoring androstenone levels in boars by direct immunochemical analysis of serum samples. *Meat Science, 61*(2), 193–197.

Tuomola, M., Vahva, M., & Kallio, H. (1996). High-performance liquid chromatography determination of skatole and indole levels in pig serum, subcutaneous fat, and submaxillary salivary glands. *Journal of Agricultural and Food Chemistry, 44*(5), 1265–1270.

Verheyden, K., Noppe, H., Aluwe, M., Millet, S., Vanden Bussche, J., & De Brabander, H. F. (2007). Development and validation of a method for simultaneous analysis of the boar taint compounds indole, skatole and androstenone in pig fat using liquid chromatography-multiple mass spectrometry. *Journal of Chromatography A, 1174*(1–2), 132–137.

Wesoly, R., Jungbluth, I., Stefanski, V., & Weiler, U. (2015). Pre-slaughter conditions influence skatole and androstenone in adipose tissue of boars. *Meat Science, 99*, 60–67.

Wu, J. T. (2001). The development of a staggered parallel separation liquid chromatography/tandem mass spectrometry system with on-line extraction for high-throughout screening of drug candidates in biological fluids. *Rapid Communications in Mass Spectrometry, 15*(2), 73–81.

Zamaratskaia, G., Babol, J., Andersson, H., & Lundström, K. (2004a). Plasma skatole and androstenone levels in entire male pigs and relationship between boar taint compounds, sex steroids and thyroxine at various ages. *Livestock Production Science, 87*(2–3), 91–98.

Zamaratskaia, G., Babol, J., Madej, A., Squires, E. J., & Lundström, K. (2004b). Age-related variation of plasma concentrations of skatole in relation to androstenone, sex hormones, triiodothyronine and IGF-1 in six entire male pigs. *Reproduction in Domestic Animals, 39*, 168–172.

Zamaratskaia, G., & Squires, E. J. (2009). Biochemical, nutritional and genetic effects on boar taint in entire male pigs. *Animal, 3*(11), 1508–1521.